Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Other PCR Procedures

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Description

Molecular toxicology is an emerging discipline that utilizes molecular and cell biology to understand how drugs and chemicals result in their unwanted effects.**PCR Protocols in Molecular Toxicology** is a practical guide to the use of polymerase chain reaction (PCR) to help examine, on a molecular and cellular level, how toxic responses are manifested. It offers a basic understanding of PCR and its optimization, as well as describing specific, high-impact areas of molecular toxicology and recent advances. The following techniques are described in detail:

- Quantitative reverse transcriptase PCR and methods to examine gene expression

- Differential display cloning
- Cloning and library screening by PCR
- Genotype and polymorphism analysis of drug and toxicant metabolizing enzymes
- Basic, non-PCR based molecular biology methods

PCR Protocols in Molecular Toxicology will aid both novices and experienced PCR practitioners in using PCR to its fullest potential.

Features

- Provides detailed instructions on how to adapt procedures to various issues of toxicological interest
- Discusses how to fully utilize the powerful techniques of PCR
- Addresses the arrangement of work space, design of primers, optimization of the basic PCR, and troubleshooting tips
- Tells how you can utilize the Internet and World Wide Web to perform PCR experiments
- Contains appendices with lists of suppliers and other resources, including online catalogs and materials available through the Internet

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I. Introduction

The polymerase chain reaction (PCR) has changed many of aspects of day-to-day laboratory work. For example, our lab uses PCR to quantitate mRNA levels (RT-PCR), to generate cDNA fragments for cloning or subcloning, for the identification of bacterial colonies, DNA sequencing and recently for differential display cloning (DD-PCR) and library screening. In addition, PCR has classically been an important tool for the identification of mutants (PCR-SSCP) and polymorphisms (PCR-RFLP), discovery of unknown sequencing flanking known regions (5' and 3' RACE, inverse PCR) and other important molecular techniques ugeneration of ssDNA). In this workbook basic principals of primer selection and quantitative reverse transcriptase PCR will be stressed. However, I will also try to include other techniques and tips we have discovered.

There are many basic considerations to being an effective PCR'er. First, your laboratory needs to be properly equipped. The thermocycler may become the workhorse of the lab and can be used for PCR as well as other enzymatic reactions (i.e. restriction enzymes, ligases, polymerases). We have always used the Perkin Elmer 9600 thermocycler as this was the top-of-the-line model. Although there probably is no better model on the market, there certainly are cheaper units available. Make sure that any PCR machine you buy is fully programmable (can do the cycle profile as well as certain advanced options such as "touch-up" or "touch-down" and increasing elongation time/cycle), can handle 96-well plates and has a

heated lid for oil-free operation. The latter consideration will increase the cost of the thermocycler, but it is definitely worth the extra money.

In addition to having a PCR machine you will also need to configure your lab space differently. You will need a room dedicated to the assembly of PCR reactions and a separate lab for the examination of amplification products. NEVER ALLOW TUBES CONTAINING AMPLIFIED PCR PRODUCTS TO BE OPENED IN THE SAME ROOM AS THAT USED TO ASSEMBLE PCR REACTIONS. The best analogy that I have heard regarding the problem of PCR contamination is to compare your lab space to that occupied by a swimming pool. If one PCR reaction is emptied into an olympic size swimming pool and allowed to mix and a 5 ul aliquot removed, you would have about 500 molecules of your previous PCR product! You can see why there is concern over contamination. The electrophoresis chambers and PCR purification products are placed in a separate room from the thermocycler. Also, you will need dedicated pipetters for PCR assembly, for PCR product handling (which will be in the other room) and possibly for the handling of internal standards. The pipetters should be disassembled periodically and either uv irradiated or bleached. All tubes and pipette tips should be autoclaved while the tube retainer and trays should be bleached (10%) after every use. Other pieces of equipment necessary are a heat plate with a 96-well block set at 85 C, aerosol barrier pipet tips and a vortexer conveniently located next to the thermocycler and of course electrophoresis units. Optional equipment include freezer boxes for enzymes, a picofuge and if you are really paranoid about contamination, a PCR chamber with a uv light.

The second consideration before embarking on your quest to being a top-notch PCR'er is to familiarize yourself with the concepts of how the polymerase chain reaction works in general and specifically how quantitative aspects are confused by tube-to-tube variability. In Figure 1 below I have included a very simple description of how PCR works. Of course, the primary reference source is PCR Protocols: A Guide to Methods and Application (Innis et al, eds). I strongly suggest that this book be made available to anyone who wants to learn PCR. In addition these techniques and our basic protocols are constantly changing. In order to stay on top of all the new advances, I would suggest that a technical journal such as Biotechniques or PCR Methods and Applications be browsed frequently. More detailed understanding of computer analysis of DNA and mRNA sequence and structure and primer selection is required of at least one person in the proposed studies. Many of the computer search techniques discussed below are specific to the way our lab is connected to the GenBank database. You will want to adapt to how you currently access nucleotide sequences. In addition, we are currently using a program called Lasergene by DNAstar (Madison, WI) to find primer sequences. I will try to point out the criteria required to select good primers so that you can use whatever software you please.

The last item required to be a good PCR'er is patience. This technique is very sensitive to timing (how quickly reaction are assembled), season (summers are often prone to crashes), various forms of contamination and intermittent bouts of stress and tedium. After a series of failures, it is often best to walk away from PCR and indulge in your favorite vices for a period of time. Although PCR is considered science, (it is simply a glorified enzyme assay), there is a lot of art involved and a little luck never hurts either. Learn the basics and cross your fingers.

Figure 1. Principle of PCR and reverse transcriptase PCR

To view Figure 1, click here

II. Searching the Database for Sequence of Interest

A. Basics

The way in which you find your sequence of interest depends on your familiarity with programs such as GCG (Genetic Computing Group, Madison, WI), MacVector, Lasergene or network gophers. I have shown in detail how to find a DNA sequence using GCG as this is probably the most powerful single tool for sequence analysis and is available at most universities. The search is often made easier if you know an accession number for your gene, the author of the paper in which it was discussed or you can type in a sequence string to compare to the data base. Often trivial names make it difficult to obtain the correct sequence. Being familiar with database searches and retrieval will also be necessary in order to pick primers for a gene within a large family (i.e. if you want primers specific for CYP1A1 versus CYP1A2 you will need to compare the sequences to find regions of divergence) and to test the specificity of your primer set. For GCG, a complete guide to the program is available via the Genetics Department at UW or can be downloaded from the internet. Beware, the manual is written assuming that you already know how to use GCG and therefore do not need the manual! Have someone familiar with the program, and other database retrieval system, walk you through the search the first couple of times.

B. Searching for sequence using GCG

1. At $ prompt type "strings" or "stringsearch"

2. Select database to search. Default is GenEMBL:* which includes all nucleic acid sequences. Ro:* searches for roden sequences, ov:* for other vertebrates, pr:* for primates, etc.

3. Search for Names (Option A)

4. Type name or string you want to search (i.e. if you want a list of cyt Chrome P450 enzymes type "cyt Chrome" or "CYP".)

5. Give name for output file (i.e. CYP1a1.strings) and .

6. To view output type "type xxx.strings ". To print output type "type xxx.strings ".

7. Find the file that contains the sequence of interest (i.e. rat cyt Chrome P4501A1, mRNA). This file can then be retrieved by using the command "fetch". Type "fetch gb_ro.xxxx" or use the accession number i.e. "fetch X10245 . The fetch command will take gb_ro.xxxx and download the file as xxxx.gb_ro.

8. View this file by using the command "type" i.e. "Type xxxx.gb_ro"

9. Download sequence to disk or PC. Use the kermit program with your communication program or FTP. The mode of transfer may also depend on the type of primer analysis program you are using.

III. Primer Selection

A. Basics

Before you start to search for primers you should try to learn as much as possible about the structure of the gene in question. It is good to know the location of introns, coding region and any other points of interest such as splice sites and alternative splicing, polymorphisms and mutations. Often this is found in the print-out from GCG or by doing a routine literature search. Unfortunately, often the gene of interest is closely related to another gene or family such as cytochromes P450 or steroid hormone receptors. In order to pick specific primers, you may have to compare the similar sequences and pick primers in a

dissimilar region. The GCG command called "gap" will allow you to compare genes (i.e. $gap cypla1.seq cypla2.seq). If you fear having DNA contamination in your RNA, primers which span an intron should be used. In general, primers which are near the 3' end of the mRNA and in the coding region work the most consistently.

The criteria used to select primer pairs varies from researcher to researcher. Some of the rules suggested include (a) primers with similar melting temperatures with 40-60% GC content, (b) primers flanking intron-exon border, (c) size of PCR product between 100-1000 bp, (d) primers 18-22 nucleotides, (e) GC at 3' end of primer, (f) no primer should contain more than 4 contiguous base pairs of homology to itself or counterpart and (g) the PCR product should have a predicted melting temperature (Tm) of 76-82 C. Of course, primer selection is often empirical and none of these rules are written in stone. For example, we find that having a GC at the 3' end of a primer is not necessary, many of our primers do not span an intron and the GC content of our primers and PCR product is rarely 50-60%. The most important criteria are (1) the 3' end of the primer should be highly specific, (2) no primer should contain more than 4 contiguous base pairs of homology to itself or counterpart, (3) product length 100-500 bp, (4) primer Tm difference 2-5 C and (5) primers should be at the 3' 50% of the cDNA.

When looking for a primer selection program make sure that it takes points 1-4 into consideration. In the following section I use the Lasergene program as a specific example of how to choose primers. MacVector also has a primer selection and rumor has it that there are GCG programs and shareware/freeware that can be used. Most of the equations and algorithms used can be found in the literature [Primer selection: Rychlik and Rhoads (Nucl. Acids Res. 17: 8543-8551, 1989), Hillier and Green (PCR Methods and Applications 1: 124-129, 1991), Lowe et al. (Nucl. Acids Res. 18: 1757-1761, 1990); Annealing temperature: Rychlik et al. (Nucl. Acids Res. 6409-6412); Helix stability, Frier et al. (PNAS 83: 9373-9377, 1986) and Rychlik et al. (PNAS 84: 945-949, 1987)]. In addition, free or cheap copies of PCR selection programs are available (OSP; see Hillier and Green PCR Methods and Applications 1: 124-128, 1991; see Lowe et al., Nucl. Acids Res. 18: 1757-1761, 1990, $50) although we have not been able to test these. Incidentally, most programs were developed based on the paper by Rychlik , if you are interested in the algorithms involved.

B. Using the Primer Selection Program

1. Make sure that you have the correct sequence loaded onto your computer. Double check so that stupid mistakes can be avoided.

2. Retrieve sequence of interest into your analysis program. Depending on the program being used this can be done in several ways. Make sure that the correct sequence is being searched.

3. Adjust the search conditions. Once again this may be done in several ways, depending on the program. Keep in mind that we prefer to pick primers between 17-21 bp which are found in the last half of the cDNA and result in a product of 100-400 bp. Change the programs default conditions to reflect this bias. The rest of the conditions are usually kept as is, for now.

4. Select compatible primer pairs. Most of the newer programs will give you a list of compatible primer pairs and may also rank them based on certain criteria. Take the primer pair that is ranked the best, if it meets all of the other extraneous conditions (i.e. spans an intron or splice site; is in an area of divergence with a related genes etc.)

5. Often the search parameters need to be altered because the program did not find an appropriate PCR pair. Now you must go back and change the search conditions. Try the following hierarchy for adjusting

these conditions
a. Adjust location: Pick a wider range to examine and allow for longer products
b. Change primer length: Usually easier to find compatible primers if they are shorter
c. Allow 3 contiguous base to pair with itself (or if your program is set for 3, allow for 4)
d. Allow the difference in melting temperature to vary by greater than 10 degrees.

If you still cannot find a suitable pair, you may have to pick primers by hand. Do the best you can, but be ready to do a lot of optimizing later.

C. Computer Analysis of Primers

After a primer pair is chosen by whatever means available, we generally make sure that there is no gross homology with other sequences. This will not ensure that the primers will work, but it will indicate if major problems exist. Once again, how you do the computer search depends on your connections. I have been using BLAST, which is also available as an e-mail type of search to NCBI. (Details on the latter can be obtained by email to NCBI) or through the internet. I have demonstrated how to use the GCG method, but highly recommend the NCBI Blast Homepage. It is very intuitive and has help screens

Here is the link for the blast server. Blast Search

1. It is usually a good idea to check your primers for possible annealing to known sequences. Both the forward and reverse primers will have to be significantly similar to the same sequence in order for there to be a potential problem.

2. Log on to the VAX and enter GCG as discussed above. At the $ prompt type "seqed" to enter the sequence editor.

3. Type the name of the primer you wish to enter i.e. "xxxxfp.seq" followed by "z". Type in any header you want followed by z to get to the editing line. Type sequence of the forward primer in the 5' to 3' direction then "Z". At the : prompt type "exit" (DO NOT type "quit" or your sequence will not be saved).

4. Repeat for reverse primer (i.e. xxxxrp.seq).

5. To search if the primer sequences are similar to known sequences use the command BLAST. At the $ type "BLAST" and enter the name of the query sequence (i.e. "xxxxfp.seq"). To accept the default search parameters hit twice. When prompted for a database, reply with the database from which the original sequence was found, i.e. ro:* or ba:* followed with . You will also be asked for the number of scores to view. Usually the 10 best scores are more than enough. Name the file "xxxxfp.blast". It will take a while for the search to be complete; however if the /batch qualifier was used, you can now search with the reverse primer.

6. To view results type "type xxxxfp.blast". The sequence that you picked the primers to study should be seen with both blast searches. No other sequence should be observed in both lists. Pay particular attention to those sequences which have high homology with the 3' end of your primer sequences.

IV. Optimizing Primer Set

A. General Considerations

There are four main parameters which need to be optimized for each primer set: 1) Mg++ concentration, 2) annealing temperature, 3) pH and 4) number of cycles.

In general, the primers picked by the typical programs have little magnesium effects. Also, the optimal annealing temperature calculated tends to be lower than optimal. Start with 30 cycles for the first trial and adjust from here. The general recipes for the cDNA reaction and for PCR are shown in the accompanying appendices.

B. Optimizing Mg++

1. Set-up cDNA reaction as discussed in Appendix I. Run 6 samples minimum for each primer pair.

2. While cDNA reaction is proceeding, make PCR mix with no MgCl2, as shown below. Heat to 85 C.

	1X
ddH2O	16 ul
10X buffer	3 ul
forward primer	0.6 ul
reverse primer	0.6 ul
Taq	0.25 ul

3. Add the following to the cDNA reaction (at room temperature).

Tube #	ul MgCl2	ul ddH2O	Final[Mg] (mM)
1	0	10	1
2	2	8	2
3	4	6	3
4	6	4	4
5	8	2	5
6	10	0	6

4. Place tubes in heat block. Add 20 ul PCR mix (hearted to 85 C) to each tube and cycle as suggested in Appendix I using the annealing temperature suggested by the analysis program

5. Separate PCR products as described in Appendix III. Compare the intensity of bands and the appearance of artifacts relative to MgCl2 concentration.

C. Optimizing Annealing Temperature and Troubleshooting

1. Start with temperature suggested by the computer. If your program does not suggest an optimal annealing temperature, try the following calculation.

T(opt) = 0.3 (Tm(primer)) + 0.7 (Tm(product))-14.9

2. If too many bands, add a "touchdown" (see Appendix I), decrease the cycle number slightly, or increase the annealing temperature +2C from suggested. Continue increasing annealing temperature up to a maximum of approximately 62 C.

3. If very weak bands, add a "touchup", increase cycles slightly or decrease annealing temperature -2C from suggested. Continue decreasing annealing temperature to a minimum of approximately 45 C.

4. If using primers derived from sequence information in one species to amplify mRNA in another species try suggested temperature. If this is does not work, try step 3.

V. Making of a PCR Internal Standard

A. Basics

It is possible to make RT-PCR quantitative, so do not listen to non-PCR'ers. However, you need to find a way to negate the tube-to-tube variability that is inherent in the amplification process. The only good way to do this is to add an internal standard (IS) each tube. There are many ways to make internal standards; the method shown below has several advantages in that it is adaptable to any primer sequences and is easy to perform. How you make an internal standard is not as important as its properties. First it should be amplified with the same efficiency as the cDNA being quantified. This is generally done by having the IS and target with the same primer recognition sequences and by making the two PCR products of similar length. Also, you need to resolve the IS PCR product from the target PCR product. This may be done by changing a restriction enzyme site in the IS or by making the IS of a slightly different length. Another factor when using an IS for RT-PCR is to start with a RNA IS as well to negate variability in the cDNA synthesis. The method used in our lab does fulfill these requirements, as you will see.

B. Design of Internal Standard Primers

1. For basics of internal standard synthesis see Figure 1 of Vanden Heuvel et al. (Biotechniques). The design of the internal standard is: Forward Primer--T7 promoter, target forward primer, spacer forward primer; Reverse Primer--spacer reverse primer, target reverse primer, poly(T)15.

NOTE #1: The spacer primer sequences are designed so that ANY sequence can be inserted. The key is to find a primer pair (the spacer primers) that will ultimately result in a PCR product that is different from that of the target. In the Biotechniques paper we used human GSTMu as our linker primers. We have also used interferon-g and b-globin sequences to make primers. The key is to find primers that work and they give the appropriate size product. Generally, we have browsed through commercial catalogs to find sequences.
NOTE #2: The primers used in the making of the IS are quite long (around 60 bp) but only the 3' end (that containing the linker or spacer primers) will anneal and amplify. The rest of the primer will be incorporated into the PCR product.

2. It is possible to design internal standards with more than one gene per internal standard. Basically you can have two genes per internal standard primer and you can do two rounds of application. For example, round one forward primer Target 2 FP, Target 1 FP and Spacer FP; round one reverse primer Spacer RP, Target 1 RP and Target 2 RP. Second round forward primer, T7, Target 3 FP, Target 2 FP. Second round reverse primer, Target 2 RP, Target 3 RP and PolyT. Much more thought goes into designing these primers.

3. We use human genomic DNA as a source of template for making internal standard to use for rat genes. It probably does not matter, but it makes us feel safer.

C. Internal Standard Amplification

1. Amplify human genomic DNA (10 ng, if this is the source of your linker primers) using the reverse and forward internal standard (IS) primers (See appendix IV). Do multiple PCR reactions for each IS. A

touch-down and 35 cycles is suggested.

2. Pool the multiple IS reactions and purify using Magic PCR Prep Purification system (Promega) or Microcon 100 spin columns following the manufacturers suggestions. This step will remove unused primers. Analyze on an agarose gel. At this point you may not be able to see a PCR band, depending on the efficiency of the primers.

3. Dilute PCR products 1:100 and amplify multiple tubes (5-6) as shown in step 1.

4. Pool and purify PCR products as stated in Step 2. Analyze PCR products on an agarose gel. If the product is not clean enough, gel purify the appropriate band. If a nice clean IS band is observed, continue with the in vitro transcription protocol as listed below.

D. In vitro transcription

1. Prepare the following mix (from Promega's Gemini II kit)

Transcription buffer	20 ul
100 mM DTT	10 ul
rRNasin	2.5 ul
rATP/rCTP/rGTP/rUTP	5 ul each
IS PCR product	45 ul
T7 RNA polymerase	2 ul

Incubate for 1-2 hr at 37 C

2. Add 2 ul RQ1 RNase-Free Dnase. Incubate for 15-30 min at 37 C.

3. Add 100 ul TE-buffered phenol. Vortex for 1 min and centrifuge at 12,000 g for 10 min.

4. Transfer upper phase to a fresh tube. Add 100 ul chloroform/isoamyl alcohol (24:1).

5. Transfer upper phase to a fresh tube. Add 50 ul 10 N ammonium acetate (pH 4.0) and 500 ul ethanol. Precipitate at -20 C for 30 min.

6. Spin at 12,000 g for 10 min. Wash with 70% ethanol.

7. Quantitate RNA using absorbance at 260 nm. [Note: to quantitate RNA use the following formula: ABS260 x 0.04 x dilution factor = ug/ul].

8. How to calculate molecules/ul of IS:

$$\frac{\text{ug/ul}}{(330\ \text{ug/umol/bp} \times \text{bp IS})} \times 6.02 \cdot \text{E } 17\ \text{mlcls/umole}$$

The 330 x bp is an approximation for the molecular weight of the internal standard. For example, a 0.1 mg/ml solution of a 400 bp IS would be 4.56 x 1011 mlcls/ul.

VI. Quantitative RT-PCR

A. Basics

The procedure we use is called "competitive" RT-PCR. However, there is no real competition in the biochemical sense as all reagents are in excess. The only "limiting" factor is the copies of cDNA in your tube in the first few cycles. However, when you look at a competitive PCR reaction, it sure looks like it is competitive. Just put your brain on hold and accept the fact that it "looks" like something is limiting. Really, it is a kinetic issue and not a competition for substrates; the higher the concentration of IS, the higher the probability that the primers will find it instead of the target. The internal standards should amplify at a similar rate to the target if they are of similar lengths. This is due to the fact that both messages have the same primer sequences and are amplified in the same tube. For a basic diagram of how to set up a competitive reaction RT-PCR, see Figure 2 of Vanden Heuvel et al. (Cancer Research). Once again, remember that the IS are rcRNA molecules which are spiked into the RNA prior to RT. Also note that at this point the primers used to make the IS are no longer needed.

Figure 2. Basic RT-PCR techniques

Dilute the RNA samples to 0.05 ug/ul in DEPC-treated water. The RNA can be extracted using any conventional method that effectively removes the DNA. We are currently using a product that works quite well, called Tri-Reagent (Molecular Research Center). The stock RNA is stored at -70 C while the diluted RNA may be stored at -20 C. There is no need to ethanol precipitate the diluted samples. The diluted stock is now ready for RT-PCR. The first thing to do is to estimate how much target mRNA is in the samples (see B below) followed by quantitation using either method presented (C and E).

B. Range Finding

1. In order to accurately quantitate mRNA levels using RT-PCR, an approximation of message levels is often necessary. Also, quite often a large difference between treatment groups may be evident after this range finding experiment and true quantitation may be irrelevant.

2. Dilute the internal standard to 1E10 molecules/2 ul and do 10-fold dilutions of this stock in DEPC-treated water.

3. Make a cDNA mix with enough for 8 aliquots of your samples, plus some extra. For example if doing range finding on 2 samples and 8 IS concentrations make a stock enough for 24X instead of just 16X.

4. Add 2 ul RNA sample (100 ng) to each of 8 tubes. Add 2 ul of serial diluted IS to each tube (10E9 to 10E2 mlcls/2 ul). Add 16 ul cDNA mix to each tube.

5. Run cDNA reaction and PCR cycle as optimized previously. You may also want to run a blank sample as well (no RNA, no IS).

6. Separate PCR fragments on agarose gels. Where is the cross-over point?

7. The range finding experiments may tell you whether a treatment has a profound effect on mRNA levels for your gene (i.e. 10-fold increase). It will also tell you the IS concentration to focus a narrower range finding experiment. You may need to use a different IS range for treated vs non-treated samples.

C. Quantitation using method of Gilliland

This method is discussed in PCR Protocols (Innis et al., eds). When few samples are to be examined,

this is the best method to use and gives the most convincing photographs and data. However, it requires much more patience than method E shown below and is more expensive, time consuming and generally mind-numbing. Good place to start for the inexperienced PCR'er.

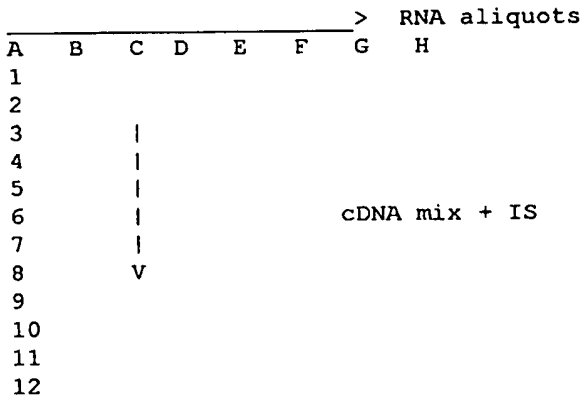
1. The range finding will dictate the IS concentration range for quantitation. For example if the cross-over point is between 10E5, to 10E6 mlcls, use the following IS concentrations: 10E7, 10E6, {8, 6, 4, 2, 1}x10E5, 10E4 mlcls/2 ul.

2. Automate the PCR as much as possible for quantitation. Run as many samples as possible from the same cDNA and PCR mixes. Also, run the maximum number of samples per tray if possible. Stock cDNA and PCR mixes (without MMLV-RT and Taq) may be stored at -20 C without any problem.

3. Make stock mixes based on the number of samples you want to analyze for a given gene. For example if you have 36 samples, make enough mix for 3 full trays. Store the excess. Make stock cDNA mix, enough for 112 samples/tray (creates room for error). Divide into 224 ul aliquots.

4. Similarly, make PCR stock enough for 112 samples/tray and divide into 3.36 ml aliquots. Store the extra mixes.

5. Note the basic design for PCR tray shown below. Add 28 ul IS to each tube of cDNA mix and label A-H. Pipet 2 ul of RNA sample into each of 8 tubes (shown as rows 1-12). Add 5 ul MMTVRT to cDNA mixes (A-H). Pipet 18 ul of this mix to the RNA samples going down the columns A-H. For example the lowest IS concentration, H, is placed in tubes 1H, 2H, 3H, etc. Always pipet IS from lowest to highest concentration to avoid carry-over problems. Change tips with each tube.



6. Run cDNA reaction as per usual. Heat PCR mix to 85C for 5-10 min before adding Taq. Place cDNA samples in heat block. Add PCR mix as quickly as possible. Remember, pipet from low IS to high IS when possible (i.e. add PCR mix to 1H, 1G, 1F, etc). Change tips with each tube. Run optimized PCR program.

7. It is important that the tubes with PCR products are not opened in the room where the PCR is set-up. Take tubes to a separate room and add 5 ul 10X sample buffer. Run gels as usual and take a photograph with film that produces a positive image (Polaroid Type 66).

8. Proceed with data analysis as shown below if the differences are too subtle for yes or no answers.

D. Scanning and quantitation

1. We use a pdi scanning densitometer; however, any image analysis system that can calculate volumes of spots and subtract background will work. Find the volume (density x area) or if desired the peak height for each spot.

2. Plot [Volume I.S./Volume RNA] vs. Molecules IS as shown in Figure 3 of Vanden Heuvel et al. (Cancer Res.). Use linear regression to draw a line through the data. [Note: often a data point is not in the linear region of the curve and often the curves are not always perfect. If a point needs to be removed, it is often the highest or lowest IS ample. When possible, remove this data from all samples.]

3. The amount of target mRNA is determined by calculating the IS concentration where there is equivalent volume of IS to RNA (IS/RNA = 1). Calculate molecules target mRNA using the formula x= (1-yintercept)/slope where x=the amount of target mRNA in your sample.

E. Quantitation using one standard curve

a. Basics

This method has several advantages over that of Gilliland. First, it requires only one standard curve per plate, and this is generally a log scale of IS. Second, many samples may be run at one time, each with one concentration of IS added. We have been running each sample in triplicate, i.e. three sample #1 with 10E6 molecules IS added (from the mix) and averaging the values. However, range finding experiments must be performed to determine how much IS to spike into the tubes.

1. The general reactions are set up the same except that two mixes are made for the cDNA reaction. Make a standard curve mix and a sample mix. The standard curve mix does not contain IS but has mRNA spiked into it. The sample cDNA mix has a constant amount IS (the amount determined by range finding) but no mRNA.

2. ON ICE, pipet 2 ul of log concentrations of IS into the standard curve tubes and 2 ul of the diluted mRNA into the sample tubes.

3. Add RT to the cDNA mixes and add 18 ul of standard curve mix to the standard curve and 18 ul sample mix to the sample tubes.

4. Proceed with the cDNA reaction, PCR, gel electrophoresis and densitometry as per usual.

b. Quantitation

1. Determine the density of mRNA and internal standard for the standard curve and for samples.

2. Calculate ratio of densities as mRNA/IS ("density ratio")

3. Calculate log (mRNA/IS)

4. For the Standard Curve: Plot log (mRNA/IS) vs log (IS) and perform linear regression to determine slope and intercept.

```
log (mRNA/IS) = slope(log IS) + intercept
       Density
[IS]   ratio
```

```
1e9      .04
5e8      .11
5e7      .56
...      ...
log(mRNA/IS)=-05772 log[IS] + 4.09
```

5. Solve for mRNA/IS = 1. In this case the mRNA/IS = 1 at [IS] = 1.23 x 10E7.

6. For calculation of samples using this standard curve need to do a transformation by using the amount of IS and calculated amount of mRNA ("actual ratio")

[IS]	[mRNA]	ratio	Actual ratio	Density ratio
1e9	1.23e7		1.23e-2	.04
5e8	1.23e7		2.46e-2	.11
1e8	1.23e7		1.23e-1	.56
...	...		...	...

7. Plot log"density ratio" vs. log "actual ratio" and perform linear regression.

log ("density ratio") = slope (log"actual density")+intercept

In this case:

log ("density ratio") = 0.577 (log"actual density")+0.002

8. To calculate single point measurements of unknown concentrations use the transformed standard curve. Also note that the "actual ratio" = amount of mRNA/amount of internal standard and that for single point determinations you know the amount of internal standard added to each tube. (For example, one plate may have contained one standard curve plus 10 samples in triplicate with 10E8 molecules internal standard= "actual IS").

```
        For obtaining amount of mRNA
log ("density ratio") = slope x log("actual mRNA"/"actual IS")+intercept
log ("density ratio") - intercept = slope x log("actual mRNA"/"actual IS")
(log ("density ratio") - intercept) /slope = log ("actual mRNA"/"actual IS")
10((log ("density ratio") - intercept) /slope)=actual mRNA/actual IS
actual mRNA = actual IS x 10 log "density ratio" - intercept /slope
```

For example using the transformed standard curve above

log ("density ratio") = 0.577 (log"actual density")+0.002 and a sample with a density ratio of 0.4 and a actual IS of 1 x 108 molecules

```
log 0.4 = 0.577 (log actual ratio) + 0.002
-.4 = 0.577 (log actual ratio) + 0.002
log actual ratio = -.693
actual ratio = 0.203
actual mRNA = 2.02 x 107
```

F. Other considerations

1. It may be a good idea to quantitate a house-keeping gene mRNA as well as target mRNA. This will correct for unequal amounts of mRNA in total RNA. Good housekeeping genes are b-actin, APRT, HPRT or glucose-6-phosphate dehydrogenase. Note: b-actin and G6PD are high expression, HPRT and APRT are low expression. Standardize with a housekeeping gene which is similar to the expression of the gene of interest.

2. Since there may be some variability in the quality of RNA extraction from day-to-day, try to extract all samples to be analyzed in the same run, if possible.

3. Automation is a key. Anything you can do to add reagents faster and with more accuracy will help your RT-PCR.

APPENDIX I: Basic RT-PCR Recipes

How to use these recipes: In general, how we do RT-PCR is to calculate how many samples we want to run and add 10%. Make a cDNA or a PCR mix based on this over estimation. For example, if you are running 30 samples, make a mix for at least 33. Take the recipe below and multiply each component by 33.

cDNA Reaction **Component (and Promega Cat.#)**	**1X**
ddH2O	8.5
MgCl2 (25 mM) (#A3511)	4
10X Buffer (See Appendix II)	2
dATP (100 mM)(#U1201)	0.2
dCTP (100 mM)(#U1221)	0.2
dGTP (100 mM)(#U1211)	0.2
dTTP (100 mM)(#U1231)	0.2
Oligo(dT)15 (0.5 mg/ml)(#C1101)	0.25
rRNasin (30 U/ul)(#N2513)	0.25
Assemble on ice Pipet RNA into tubes. Add to mix:	
MMLV Reverse Transcriptase (Gibco BRL)	0.2

Note: Add MMLV RT last

Add 16 ul cDNA mix to 2 ul RNA (0.05 mg/ul) and 2 ul internal standard (or water).

Total Volume = 20 ul

Heat to 42 C for 15 min and 95 C for 5 min.

PCR Reaction **Component (and Promega Cat.#)**	**1X**
ddH2O§	22
MgCl2 (25 mM)(#A3511)§	4
10X Buffer (See Appendix II)	3
Forward Primer (10 pmol/ul)	0.6
Reverse Primer (10 pmol/ul)	0.6

```
Place tube in 85 C heat block for 3 min before adding:
Taq DNA Polymerase (5 U/ul) (#M1665)          0.25
```

---

§ Must be optimized for each primer set

Place cDNA reactions in 85 C heat block for 2 min before:

Add 30 ul PCR mix to the 20 ul cDNA reaction

Typical Cycle Program (for Perkin Elmer Cetus Model 9600) Standard

```
Denature          94C  4 min

                    Cycle (30X)
Denature          94C  15 sec
Anneal            55C  30 sec
Elongate          72C  30 sec

Elongate          72C  5 min
Store               4C
```

"Touchdown"

```
Denature          94C  4 min

                    Cycle (10X)                          Cycle (25X)
Denature          94C  15 sec                            94C  15 sec
Anneal            65C  30 sec (decrease 1C/cycle)        55C  30
Elongate          72C  30 sec                            72C  30

Elongate          72C  5 min
Store               4C
```

APPENDIX II: Buffer and Reagent Compositions

10X PCR Buffer Composition ("Magic Buffer")

Stock Solutions:

Note: All reagents should be of the highest grade and dedicated to RNA work (i.e. gloves only) and when available purchased nuclease-free. Use sterile pipet tips and glassware.

1. Ammonium Sulfate (i.e. Sigma A-5132) 22 g/100 ml in DEPC-treated water

2. 1 M Tris pH 8.8 in DEPC-treated water

3. EDTA (i.e. Fisher S311-500) 25 mg/100 ml in DEPC-treated water

4. BSA (Nuclease/Protease/Fatty Acid Free i.e. Calbi Chem #126609) 80 mg/10 ml in DEPC-treated water (store at -20 C).

5. b-Mercaptoethanol (i.e. Sigma M-3148)

Combine the following:

1 ml Ammonium Sulfate

6.7 ml Tris

1 ml EDTA

1 ml BSA

35 ul b-mercaptoethanol

DEPC-treated water up to 10 ml

Mix well and divide into 1 ml aliquots--freeze at -20 C.

PCR Primers

Dilute stock primers to 100 pmol/ul in ddH2O and store at -70 C

For a working stock, dilute to 10 pmol/ul and store at -20 C

10X TBE

324 g Tris into 1.5 L ddH2O

55 g Boric Acid

18.6 g of Na2EDTA

Stir and fill to 2 L

Filter through a Whatman #1 or equivalent

10X Loading Buffer

Add 10 ul stock bromophenol blue (0.1%) to 1 mL 30% Ficoll in TE

Agarose Gels

Mid-Sized Gels

Dissolve 2-4 g NuSieve 3:1 in 100 mL 1X TBE (Note: This is NOT the 10X). Microwave until completely dissolved. Let stand 10 min. Add 10 ul stock ethidium bromide (10 mg/ml), mix and pour gel. Let harden at least 30 min before gel is run. Submerge in 1X TBE running buffer and remove combs. Run at 80-100 V for approximately 1 hr.

Mini gels

Adjust to: 40 mL TBE, 4 ul EtBr, run at 120 volts for 2 hr Metaphor gels:

Divide percentage of agarose by one half. After gel has hardened, place in refrigerator for 1/2 hr. Run as described above. Sample Preparation

After the PCR reaction is done, remove from the assemble room into the electrophoresis room. Add 5 ul loading dye per tube. Mix by vortexing. Load 15 ul per lane.

APPENDIX III: Standard PCR

Internal Standard PCR

Component	1X
ddH2O	27
MgCl2	6
10X Buffer	5
dATP	0.2
dCTP	0.2
dGTP	0.2
dTTP	0.2
Forward IS Primer (10 pmol/ul)	3
Reverse IS Primer (10 pmol/ul)	3

Assemble at room temperature
Pipet DNA into sample tubes
Place at PCR mix 85 C for at least 5 min. before:

Taq DNA Polymerase	0.2

Add 45 ul mix to 5 ul DNA sample. The DNA sample should be diluted to 10-100 ng/ul. Lately we have been using 100 ng genomic DNA/tube.

Run the PCR program as suggested in Vanden Heuvel et al. (Biotechniques).

Note: This recipe is also used for the following applications:

Clone Check

Take a toothpick stab of a bacterial colony and place in 50 ul ddH2O. Heat at 95 C for 10 min. Use 5 ul of this solution as DNA source. Prepare recipe as stated above.

PCR Cloning

Use 5 ul of DNA, cDNA or plasmid DNA as template. Usually cloning primers have restriction enzyme sites on their 5' end and are less efficient than standard length primers. Prepare recipe as stated above.

APPENDIX IV: References

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